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The evolution of solid tumors involves acquisition of genetic abnormalities, which result in changes in both the set of genes expressed and the relative levels of gene expression. A variety of methods are available for ascertaining and comparing gene expression levels between normal and malignant cells. However, the increasing number of candidate genes whose expression needs to be evaluated for prognostic, diagnostic, therapeutic, or research purposes will require obtaining material from numerous tissue sections. Therefore this proposal is motivated by the need for more effective use of clinical specimens, and will address the problem of obtaining sufficient and cell type specific mRNA from clinical breast tumor specimens. This will entail adapting/developing a new approach to archiving the repertoire of genes expressed in normal, pre-cancerous and malignant breast epithelia. In this project, the capability will be developed to isolate and amplify with fidelity total mRNA from small numbers of microdissected cells of histologically defined types. Realization of these objectives will allow, in the future, development of a resource, consisting of amplified mRNA populations from individual cells from normal and tumor material, that can be used for evaluation of the prognostic, diagnostic and/or therapeutic importance of genes expressed in breast cancer.

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Donna A Alberton 10/26/98

PI - Signature Date

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INTRODUCTION:

The evolution of solid tumors involves acquisition of genetic abnormalities, which result in changes in both the set of genes expressed and the relative levels of gene expression. Therefore it is desirable to be able to both characterize and compare the levels of expression of particular genes in normal and tumor cells. Currently, assays of gene expression are carried out on mRNA isolated in bulk from tissue specimens, or at the individual cell level by *in situ* hybridization or immunohistochemistry. However, neither approach will meet all the needs of the research and clinical communities. Conventional mass biochemical extraction procedures are not appropriate in breast cancer, because extraction of mRNA from single cell types is difficult due to the intermingling of epithelial and stromal components, and the fact that the amount of malignant or pre-malignant tissue available in the specimen is small. In addition, very few cells in the specimen make up the normal ductal epithelium. Cell type-specific gene expression can be visualized in tissue sections, but there are now increasing numbers of candidate genes to be evaluated for prognostic, diagnostic, therapeutic, or research purposes, and expression analysis of all these genes will require numerous individual tissue sections. Therefore this proposal is motivated by the need for more effective use of clinical specimens, and will address the problem of obtaining sufficient and cell type specific mRNA from clinical breast tumor specimens for analysis of gene expression in normal and diseased tissue. This will entail adapting/developing a new approach to archiving the repertoire of genes expressed in normal, pre-cancerous and malignant breast epithelia. Procedures used to obtain gene expression profiles from single neurons (Eberwine *et al.*, 1992) will be adapted for use with clinical breast cancer specimens, allowing amplification of the mRNA repertoire from small numbers of cells from normal ductal epithelium, DCIS and invasive carcinoma. The procedure involves synthesis of first strand cDNA *in situ* from tissue sections. The cDNA synthesis is primed from the 3' end of the mRNA using an oligo-dT primer incorporating the promoter binding site for T7 polymerase so that subsequently linear amplification of this small quantity of cDNA microdissected from the tissue can be accomplished by *in vitro* transcription using T7 RNA polymerase. The procedure can provide an estimated 10^6 fold amplification of the starting material and if applied to amplification of breast epithelia, could provide enough material for multiple and diverse assays of gene expression and/or for the generation of cDNA libraries. Therefore the purpose of this proposal is to develop the capability to isolate and amplify with fidelity total mRNA from small numbers of microdissected cells of histologically defined types. We will then apply these procedures to obtain expression profiles for cells representing normal epithelium, DCIS and invasive carcinoma from frozen and paraffin embedded sections of tumors. Our objectives are to: (1) Demonstrate linear amplification of high complexity RNA from a homogeneous population of cells; optimize the techniques to maximize the amount and complexity of amplification that can be obtained while preserving relative copy number of different mRNA species, and (2) apply these techniques to amplify mRNA from microdissected cells from frozen and formalin fixed sections containing normal ductal epithelial cells, DCIS and invasive carcinoma, and use this material to obtain expression profiles for these different cell types. Realization of these objectives will allow development of a resource, consisting of amplified mRNA populations from individual normal and tumor-specific material, that can be used for evaluation of the prognostic, diagnostic and/or therapeutic importance of genes expressed in breast cancer.

BODY:

A. Reporting Period

The work described herein was performed at Lawrence Berkeley National Laboratory and covers the period from 10/1/97 to 4/30/98. As of June 1, 1998, I took up my current position at the UCSF Cancer Research Institute. A revised Statement of Work and budget at UCSF have been accepted with a start date of 11/16/98.

Technical Objective and Statement of Work for this Period

1. Demonstrate linear amplification of high complexity aRNA from a homogeneous population of cells, the breast tumor cell lines BT474. Techniques will be optimized to maximize the amount and complexity of amplification that can be obtained while preserving the relative copy number of different mRNA species from samples of (a) bulk mRNA and (b) cell pellets that have been either frozen and sectioned, or formalin fixed, embedded and sectioned.

Task 1	Months 1-3	Grow BT474 cell cultures, isolate mRNA, measure expression levels of test genes in mRNA isolated from BT474 and hybridize to IMAGE cDNA array.
Task 2	Months 4-6	Carry out amplification on various amounts of bulk BT474 mRNA down to 0.1 pg, measure expression levels of test genes in aRNA and hybridize to IMAGE cDNA array.

B. Progress

Task 1

Our initial work has concentrated on establishing the assay systems we will use to measure the linearity and complexity of the aRNA amplification procedure. Measurement of mRNA expression in cells can be accomplished by making total cDNA and labeling that for hybridizations. This will result in the production of a DNA probe. Alternatively, a labeled RNA probe can be prepared using phage transcription systems. The aRNA amplification procedure we will be investigating results in the generation of an RNA probe. Most of our experience with microarray hybridizations has been with DNA probes onto DNA containing arrayed clones. We have demonstrated previously in model experiments that DNA sequence copy number measurements are quantitative over several orders of magnitude (Figure 1). However, in the case of the hybridization performed between an RNA probe and a DNA target, different hybridization parameters than for DNA-DNA hybridizations are operating. Therefore, it was necessary to demonstrate that RNA-DNA hybridizations in our hybridization environment can be performed quantitatively. For this purpose, we made arrays of cDNA clones for cERBB2, ß-actin, and Tau. We also obtained or made templates for these clones that contained the T7 phage promoter. Labeled RNA was then made from these constructs by reverse transcription to incorporaate fluorescein or Texas Red UTP. The labeled RNA was hybridized to the arrays to test sensitivity and specificity. Very bright signals were obtained, but unexpected cross hybridization was observed. While the exact nature of this is not fully understood, it is likely to be due to binding between short regions of CG rich sequence that occur in the region of the RNA transcript that is associated with the promoter, and is thus common to all of the cDNA. Further control

experiments of this type will be required to understand this problem fully so that reliable and accurate array measurement on full aRNA pools can be accomplished.

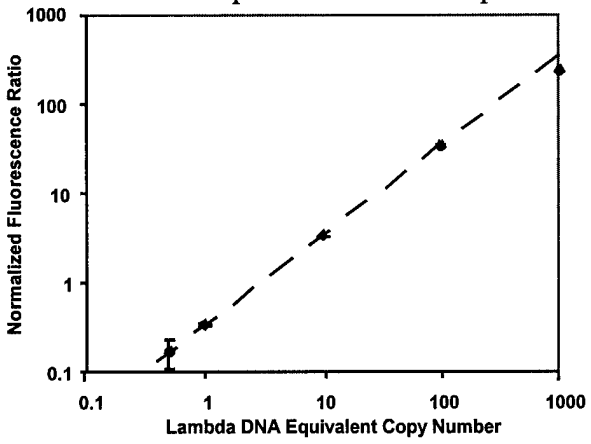

Figure 1. Fluorescence ratios as a function of lambda DNA in test genome. Test genomes contained 400 ng of human genomic DNA and 3, 6, 60, 600 or 6000 pg of lambda DNA. The reference genomes contained 400 ng of human genomic DNA and 60 pg of lambda DNA. Hybridizations were carried out to arrays containing a lambda DNA target and a human genomic DNA target. The ratios were normalized relative to hybridization to the human target. The hybridization ratios increased proportionally to the ratio of the amounts of lambda DNA from below a single copy equivlent of lambda DNA in the human genome (3 pg) to several orders of magnitude above the single copy level (6 pg).

The test cDNA arrays have also been used in hybridizations of total mRNA isolated from breast cancer cell lines. Several methods of isolating mRNA were evaluated. These cell lines had overexpression of the cERBB2 oncogene, and the signals on that target were brightest, compared to control cells, so perhaps the overexpression was detected. However, more work is needed before this interpretation is confirmed due to the potential problems noted in the previous paragraph.

Task 2

Work on Task 2 has been delayed while we validate our assay systems.

CONCLUSIONS:

Our initial work has concentrated on establishing that our protocols for fluorescence ratio hybridization to microarrays developed for use with DNA probes and targets could be used to evaluate the linearity of the aRNA amplification procedure. The results described above indicate that we can detect over expression of a gene such as ERBB2, but we have yet to demonstrate that our assay is both specific and linear when using an RNA probe. Therefore, future work will focus on these issues with regard both to hybridization to microarrays and in our other assay involving hybridization to membrane-bound targets. These experiments are the initial steps required to carry out the experiments to evaluate amplification of complex RNA populations that preserves relative copy number amongst the various species.

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